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Dryopteris protobissetiana (Dryopteridaceae), a New Diploid Sexual Species of the *Dryopteris varia* Complex (Subg. *Erythrovariae*, Sect. *Variae*) from Yakushima, Kagoshima, Japan

Kiyotaka Hori[1], Atsushi Ebihara[2], Narumi Nakato[3] and Noriaki Murakami[1]

*[1]Makino Herbarium, Tokyo Metropolitan University, 1-1 Minami-osawa, Hachioji, Tokyo 192-0397, Japan. *hori-kiyotaka@ed.tmu.ac.jp (author for correspondence); [2]Department of Botany, National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba, Ibaraki 305-0005, Japan; [3]Narahashi 1–363, Higashiyamato-shi, Tokyo, 207–0031 Japan*

The *Dryopteris varia* complex (subg. *Erythrovariae*, sect. *Variae* Fraser-Jenk.) is an apogamous fern complex. Most species of the complex are apogamous triploids; *Dryopteris saxifraga* and *D. varia*, the only known diploid sexual cytotypes in the complex, are insufficient to account for the morphological and genetic variation within the complex. In this study, we describe a new diploid sexual species, *Dryopteris protobissetiana* K. Hori et N. Murak., from southern Yakushima, and discuss its morphological characteristics, reproductive mode, and chromosome number. *Dryopteris protobissetiana* is similar to *D. bissetiana* in having slightly bullate scales and a dark green lamina surface, but it differs in having flat, serrate margins at the apex of the upper pinnae. *Dryopteris protobissetiana* is a sexual diploid with $2n = 82$, and may be a source of the genetic variation in the apogamous complex.

Keywords: Dryopteridaceae, *Dryopteris varia* complex, Japan, new species, reticulate evolution, sexual diploid, Yakushima

Dryopteris (Dryopteridaceae), one of the largest fern genera with 225 species, has a worldwide distribution (Fraser-Jenkins 1986). In Japan, 62 species, 4 varieties and 2 forms are listed by Iwatsuki *et al*. (1995). *Dryopteris* in Japan is characterized by many apogamous species. Approximately half have been reported to have apogamous reproduction, are almost always triploid and are rarely diploid or tetraploid (Takamiya 1996). Apogamous species of *Dryopteris* in Japan are difficult to distinguish from related species because they often show continuous morphological variation. Many individuals display intermediate morphology between two species, which has perplexed Japanese pteridologists.

The *Dryopteris varia* complex (subg. *Erythrovariae*, sect. *Variae* Fraser-Jenk.) is one such apogamous complex whose members are difficult to identify. Seven species, *D. bissetiana* (Baker.) C. Chr., *D. kobayashii* Kitag., *D. pacifica* (Nakai) Tagawa, *D. sacrosancta* Koidz., *D. saxifraga* H. Itô, *D. saxifragivaria* Nakai and *D. varia* (L.) O. Kuntze, have been recognized within the complex in Japan (Iwatsuki 1995, Serizawa 2009, Hori *et al*. 2014). These species are commonly characterized by having the lowest basiscopic pinnules on the lowest pinnae markedly elongate and densely scaly petioles (Iwatsuki 1995). Most species of the *D. varia* complex in Japan are apogamous triploids, rarely apogamous diploids (Table 1). Only *D. saxifraga* has been reported to be a sexual diploid. In Taiwan, Ebihara *et al*. (2014) reported a sexual diploid of *D. varia*.

Recent molecular studies have suggested that

TABLE 1. The reproductive system, ploidy level, and chromosome numbers of the *Dryopteris varia* complex.

Taxon	Reproductive mode	Ploidy level	Chromosome number	Locality	Literature cited
D. bissetiana	apogamous	2x	‘n’ = 82	Japan	Hirabayashi (1966)
		3x	‘n’ = 123	Japan	Hirabayashi (1967), Lin et al. (2003)
			$2n$ = 123	China	Nakato *et al*. (1995)
		2x, 3x	$2n$ = 82, 123	Korea	Lee *et al*. (2006)
	sexual	4x	n = 82	China	Weng (1989)
D. insularis	apogamous	2x	$2n$ = 82	Japan	Lin *et al*. (2003)
D. insularis (*var. chichimiensis*)	apogamous	3x	$2n$ = 123	Japan	Lin *et al*. (2003)
D. pacifica	apogamous	2x	‘n’ = 82, $2n$ = 82	Japan	Lin *et al*. (1992)
			$2n$ = 82	China	Nakato *et al*. (1995)
		3x	‘n’ = 123	Japan	Hirabayashi (1970)
			‘n’ = 123, $2n$ = 123	Japan	Lin *et al*. (1992)
			$2n$ = 123	China	Nakato *et al*. (1995)
			$2n$ = 123	Japan	Lin *et al*. (2003)
			$2n$ = 123	Korea	Lee *et al*. (2006)
D. sacrosancta	apogamous	3x	‘n’ = 123	Japan	Mitui (1965, 1968), Hirabayashi (1969)
			$2n$ = 123	China	Nakato *et al*. (1995)
			$2n$ = 123	Japan	Lin *et al*. (2003)
			$2n$ = 123	Korea	Lee *et al*. (2006)
D. saxifraga	sexual	2x	n = 41	Japan	Hirabayashi (1967), Mitui (1975)
			$2n$ = 82	Korea	Lee *et al*. (2006)
	apogamous	3x	$2n$ = 123	Korea	Lee *et al*. (2006)
D. saxifragivaria	apogamous	3x	$2n$ = 123	Korea	Lee *et al*. (2006)
D. shibipedis	apogamous	4x	$2n$ = 164	Japan	Hirabayashi (1974)
D. varia	sexual	2x	$2n$ = 82	Tawian	Ebihara *et al*. (2014)
		4x	n = 82	Tawian	Tsai & Shieh (1975, 1985)
	apogamous	2x	‘n’ = 82	Japan	Hirabayashi (1966, 1967, 1974)
		3x	‘n’ = 123	Japan	Mitui (1966, 1968), Hirabayashi (1970)
			$2n$ = 123	China	Nakato *et al*. (1995)
			$2n$ = 123	Japan	Lin *et al*. (2003)
			$2n$ = 123	Tawian	Ebihara *et al*. (2014)
			$2n$ = 123	Korea	Lee *et al*. (2006)

the *Dryopteris varia* complex experienced reticulate evolution (Lee & Park 2013, Hori *et al*. 2014). The most important species for investigating the reticulate evolution of the *D. varia* complex are the diploid sexual species of the complex. The diploid sexual species *D. saxifraga* and the diploid sexual form of *D. varia* are insufficient to account for the morphological and genetic variation within the *D. varia* complex. Recent molecular phylogenetic study using plastid *rbcL* and nuclear *PgiC* sequences suggest the existence of an undescribed diploid sexual species to account for the variability within the *D. varia* complex (Hori *et al*. 2014).

In the present study, we report the discovery of a previously undescribed diploid sexual species, *Dryopteris protobissetiana* K. Hori et N. Murak., from Yakushima, Japan, and describe its morphology, reproductive mode, and chromosome number.

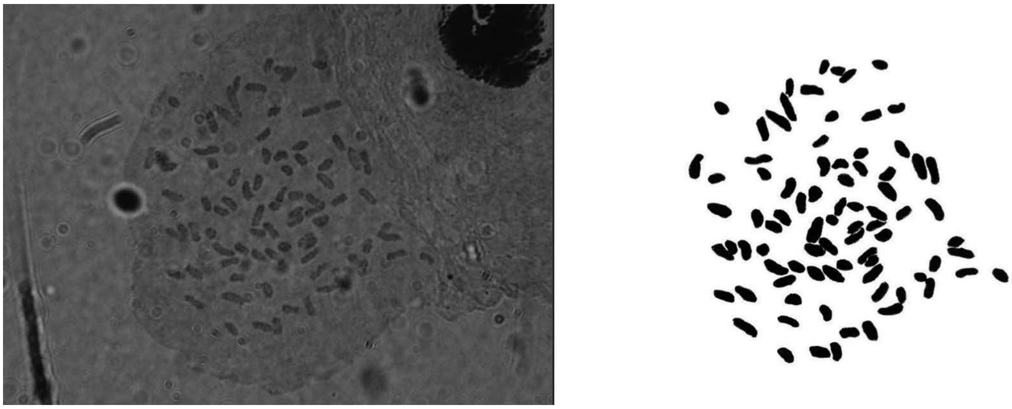

FIG. 1. Mitotic metaphase chromosomes ($2n = 82$) of *Dryopteris protobissetiana*. Scale bar: 5 µm.

Materials and Methods

Plant materials

Several leaf samples and living stocks of a new species, *Dryopteris protobissetiana*, were collected on August 7–8, 2013, from two localities in southern Yakushima, Kagoshima Prefecture, Japan. Specimens of *D. protobissetiana* were also sought in the TNS and MAK herbaria, particularly among collections formally identified as *D. bissetiana* and *D. pacifica*.

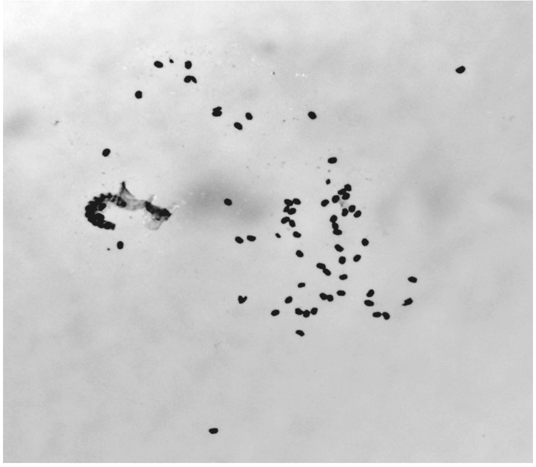

FIG. 2. Spores (approximately 64) per sporangium in *Dryopteris protobissetiana*. Scale bar: 1 mm.

Cytological observation and estimation of the reproductive mode

To observe mitotic chromosomes, root tips from one individual (*K. Hori 906*, MAK 410906) were pretreated with 0.004 M 8-hydroxyquinoline for 6 h at approximately 17–20°C. After fixation in ethanol and acetic acid (3:1) for 15–30 min, the root tips were hydrolyzed in 1 N HCl at 60°C for 1–3 min and then squashed in 2% aceto-orcein solution. Chromosomes were observed under a Nikon Optiphot microscope and photographed with a Panasonic Lumix DMC-TZ5 digital camera.

To determine the reproductive mode of each sample, the spores in one sporangium were counted. We determined samples with 64 spores per sporangium to be sexually reproductive and those with 32 spores per sporangium to be apogamous.

Ploidy analyses

Methods of ploidy analysis followed Hori *et al*. (2014). To determine the ploidy level, the DNA content of the nuclei extracted from fresh pinnae samples were measured by flow cytometry with a Cyflow Ploidy Analyser PA-II (Partec, Munster, Germany) and a Cystain UV Precise P kit (Partec). Approximately 100 mm^2 of a segment of a pinna was torn into several pieces and chopped finely with a razor blade in 0.25 mL of nuclei extraction buffer from the kit, after which 0.8 mL of

TABLE 2. Morphological differences between *Dryopteris protobissetiana* and related species.

	D. bissetiana	*D. kobayashii*	*D. pacifica*	***D. protobissetiana***	*D. sacrosancta*	*D. saxifraga*	*D. saxifragivaria*	*D. varia*
deflected scales	none	none	none	**none**	none	present	present	none
scale density	dense	sparse	dense	**dense**	sparse	dense	dense	dense
density of bullate scales	dense	sparse	rather sparse	**dense**	sparse	dense	dense	sparse
leaf texture	soft leathery	thinly herbaceous	papyraceous / leathery	**soft leathery**	herbaceous	soft leathery	soft leathery	hard leathery
leaf color	dark green	yellowish green	dark green or greenish gray	**dark green**	yellowish green	greenish gray	greenish gray	dark green or greenish gray
shape of lamina	narrowly triangular	narrowly triangular	wide triangular	**narrowly triangular**	wide triangular	oblong	narrowly triangular	pentagonal
lamina margin	incurved	flat	incurved-flat	**flat**	flat	incurved	incurved	flat
lowest basiscopic pinnule on lowest pinna	not markedly longest	not markedly longest	sometimes markedly longest	**not markedly longest**	sometimes markedly longest	not markedly longest	not markedly longest	markedly longest
serrations on margin of apex of upper pinnae	none	occationally	often	**often**	occationally	none	none	none-often
indusia	entire	entire	entire-cilliate	**entire**	entire	entire	entire	entire-cilliate

staining solution from the kit was added to the chopped tissues. The crushed tissue and buffers were filtered through a 30-μm nylon mesh (Partec). Fresh leaf tissues of *Nicotiana tabacum* L. (approximately 25 mm^2) were used as the internal standard.

Morphological comparison with other species of the Dryopteris varia *complex*

The morphology of *Dryopteris protobissetiana* was compared, mainly from original descriptions and taxonomic keys, with other species in the *D. varia* complex and in sect. *Variae*. It was also compared with species now treated as synonyms of the species of the *D. varia* complex and sect. *Variae* in Japan and China, mainly on the basis of their original descriptions and taxonomic keys. In particular, differences in the size of the lamina, the stipe, and the whole plant, shape of the fronds, color and sheen of the lamina, presence or absence of teeth at the margin of the pinnae, shape of indusia, and color and form of the scales were noted.

Results and Discussion

Ploidy and reproductive mode

Fig. 1 shows a photograph and a schematic drawing of the 82 mitotic chromosomes in one individual of *Dryopteris protobissetiana* (*K. Hori 906*, MAK 410906). All seven samples examined had 64 spores per sporangium (Fig. 2). Additional specimens of *D. protobissetiana* from southern Yakushima, all with 64 spores per sporangium and inferred to be sexually reproductive (Appendix 1), were discovered in TNS: *S. Tagane TF 293* (TNS VS 763335), Japan, Kyushu, Kagoshima, Kumage, Yakushima: Mt. Myojo, approximately 265 m altitude, Oct. 10, 2006; *S. Tagane TF 269* (TNS VS 763312), Koshima, Oct. 4, 2006; *T. Nakaike s.n.* (TNS VS 580903), Hanaage River, Aug. 17, 1982.

The size of the genome of *K. Hori 906* was 1.25 times that of the internal standard (*Nicotiana tabacum* L.) in our ploidy analyses. Considering that the genome size of *N. tabacum* is approximately 11.7 pg/nucleus (2C-value, Narayan 1987), the genome size of the diploid individuals was estimated to be approximately 14.6 pg/nucleus. The genome size of the other five samples, *K. Hori 907* (MAK 410907), *913* (MAK 410913), *914* (MAK 410914), *916* (MAK 410916) and *917* (MAK 410917), ranged from 14.6 to 16.6 pg (Appendix 1). The genome size of the triploid individuals of the *D. varia* complex was reported to range from 20.95 to 27.16 pg (Hori *et al*. 2014).

The ploidy level of all the samples of *Dryopteris protobissetiana* was therefore estimated to be diploid.

Morphological comparison with other species in the Dryopteris varia *complex*

Morphological characteristics of the Japanese species of the *Dryopteris varia* complex are summarized in Table 2. In gross morphology, *D. protobissetiana* is similar to *D. bissetiana* in having slightly bullate scales on abaxial surface of the upper fronds and dark green fronds, but differs in having the apex of upper pinnae serrate instead of entire and flat, instead of incurved margins of the pinnae (Fig. 3). *Dryopteris protobissetiana* is also similar to *D. kobayashii*, *D. pacifica*, *D. sacrosancta*, *D. saxifraga*, *D. saxifragivaria*, and *D. varia*. The latter species, however, are mostly larger, and have different morphological characteristics. *Dryopteris kobayashii* and *D. sacrosancta* have yellowish green herbaceous fronds. *Dryopteris pacifica* has papyraceous or leathery pentagonal laminae and often ciliate indusia. *Dryopteris saxifraga* and *D. saxifragivaria* have whitish green laminae, incurved leaf margins, entire margins of the pinnae and deflexed scales. *Dryopteris varia* has pentagonal laminae and often ciliate indusia. *Dryopteris pacifica* and *D. varia* have several cytotypes (Table 1) but no significant morphological differences have been observed among them.

Dryopteris insularis Kodama and *D. insularis* var. *chichisimensis* (Nakai ex H. Itô) H. Itô were classified in sect. *Variae* (Fraser-Jenkins 1986). Their closeness to the *D. varia* complex is supported by their similar morphologies as well as by the topology of the molecular phylogenetic tree (Ebihara 2011). However, *D. protobissetiana* differs from *D. insularis* var. *insularis* in not having the apex of the lamina markedly reduced and from *D. insularis* var. *chichisimensis* in having ciliate indusia.

Fraser-Jenkins (1986) included *Dryopteris hadanoi* Kurata, *D. sordidipes*, *D. shibipedis*, and *D. formosana* in sect. *Variae*. Those species, however, were reported by Ebihara (2011) to be unrelated to the *D. varia* complex on the basis of a DNA phylogenetic analysis. Furthermore, they can be readily distinguished from *D. protobissetiana* by morphological characteristics. *Dryopteris hadanoi* and *D. sordidipes* have many scales crimped to the petioles. *Dryopteris shibipedis* has flat brown scales on the petioles. The lowest pinnae of *D. formosana* are abruptly elongated. Neither *D. protobissetiana* nor other species of the *D. varia* complex display the above characteristics.

In China, 12 species have been classified in sect. *Variae* (Zhang *et al*. 2013): *Dryopteris amurensis* Christ, *D. dehuaensis* Ching, *D. formosana* (Christ) C. Chr., *D. immixta* Ching, *D. pacifica*, *D. sacrosancta*, *D. saxifraga*, *D. setosa*, *D. sordidipes* Tagawa, *D. varia*, *D. xunwuensis* Ching & K.H. Shing and *D. yongdeensis* W.M. Chu. Among them, *D. formosana*, *D. sordidipes* and *D. amurensis* have already been shown to be distantly related to the *D. varia* complex on the basis of their morphological and molecular characteristics (Iwatsuki 1995, Ebihara 2011, Hori *et al*. 2014). Accordingly, we did not compare those three species with *D. protobissetiana*.

Among the remaining five species mentioned above, which have never been recorded in Japan, we found morphological differences with *Dryopteris protobissetiana* (Zhang *et al*. 2013). *Dryopteris dehuaensis* does not have indusia. *Dryopteris immixta* has yellowish green lamina. *Dryopteris xunwuensis* has ciliate indusia and stalks 1–3 cm long on the basal pinnae. *Dryopteris yongdeensis* has russet brown scales and yellowish green, papyraceous laminae. The taxonomic treatment of *D. setosa* is complicated. In China, it is reported to have yellowish green laminae and entire or ciliate indusia (Zhang *et al*. 2013). Thunberg (1784) originally described it from Japan as *Polypodium setosum* Thunb. *Dryopteris setosa* is now treated as a synonym of *D. bissetiana* in Japan (Nakaike 1975, Iwatsuki 1995). Although, the original description did not mention the color of the laminae or the shape of the indusia, it was reported that *D. setosa* has entire pinnae and red stipes (Thunberg 1784). In these two characteristics *D. protobissetiana* differs.

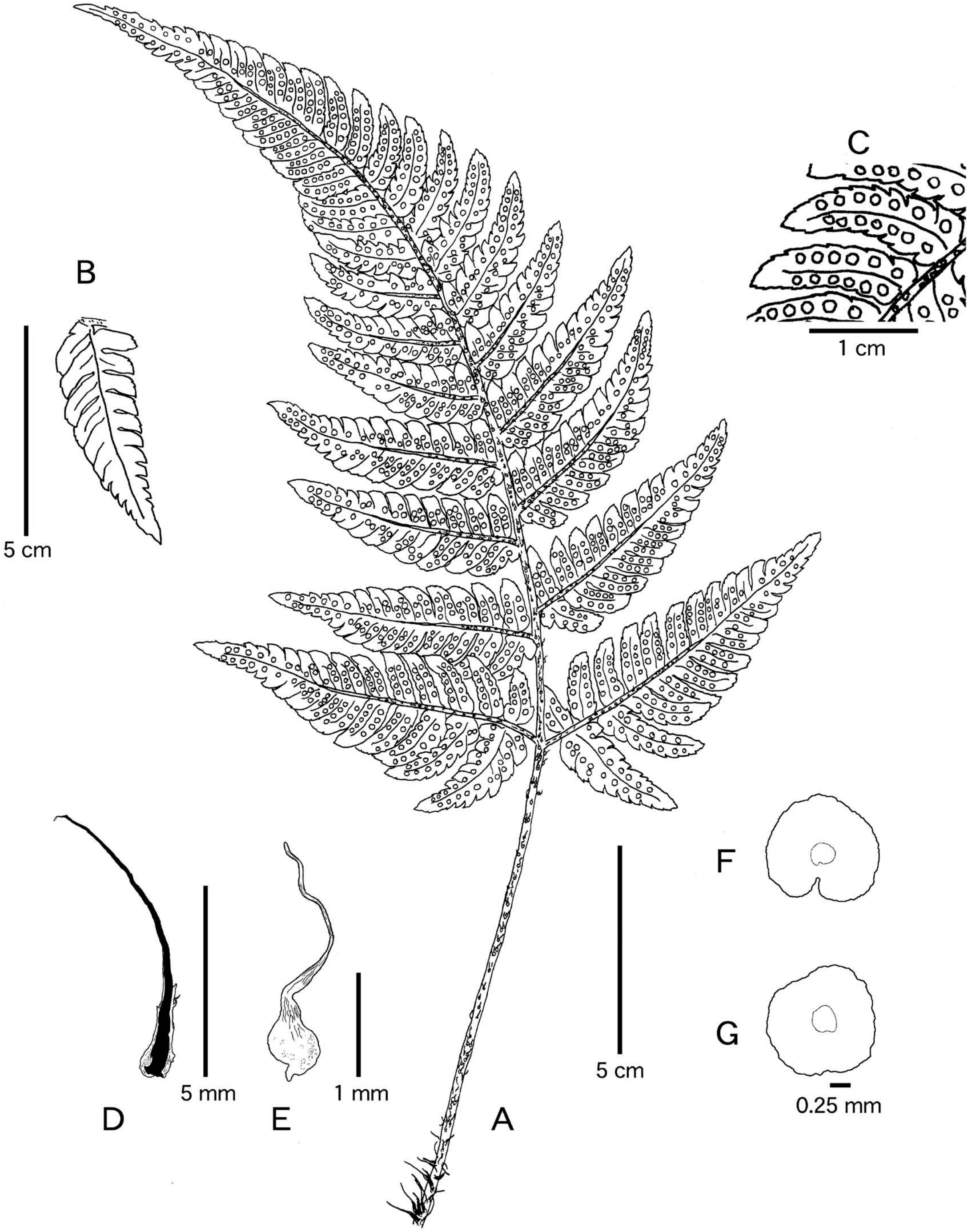

FIG. 3. Morphology of *Dryopteris protobissetiana* K. Hori et N. Murak. A: abaxial surface of frond; B: surface of upper pinna; C: abaxial surface of upper pinnae; D: scales on base of petiole; E: scales on upper rachis; F–G: indusia (drawn from type specimen: *K. Hori 913*).

There are more names that have been treated as synonyms of the *Dryopteris varia* complex in Japan, and should be compared with our new species: *D. bissetiana* var. *tenerifrons* H. Itô, *Polystichum hololepis* Hayata, *P. hololepis* var. *hikonensis* H. Itô, *Lastrea opaca* Hook., *D. yabei* Hayata, *D. matsuzoana* Koidz., and *D. ogawai* H. Itô (Ito 1939, Nakaike 1975). We can distinguish them from *D. protobissetiana* on the basis of their morphology. *Dryopteris bissetiana* var. *tenerifrons* H. Itô has ciliate indusia (Ito 1936). *Polystichum hololepis* Hayata was reported to have fronds with abruptly narrowing apices (Hayata 1915). *Polystichum hololepis* var. *hikonensis* H. Itô was reported to have ciliate indusia and fronds abruptly narrowing (Ito 1934). *Lastrea opaca* Hook. was reported to have fronds that are not glossy (Hooker 1857). *Dryopteris yabei* Hayata has ciliate indusia (Hayata 1911). *Dryopteris matsuzoana* Koidz. has 40–45-cm-long petioles and 40–43-cm-long fronds (Koidzumi 1925). *Dryopteris ogawai* H. Itô has ciliate indusia, laminae with abruptly acute apexes (Ito 1935). These morphological characteristics do not match those of *D. protobissetiana*.

Furthermore, there are many synonyms of the *Dryopteris varia* complex in China (Zhang *et al.* 2013). We can distinguish them from *D. protobissetiana* because their morphologies are distinct from that of our new species. *Dryopteris fuyangensis* Ching et P. C. Chiu, *D. lingii* Chiu, *D. paravaria* Ching et P. C. Chiu, *D. pudouensis* Ching, *D. shanghaiensis* Ching et P. C. Chiu, *D. sinobissetiana* Ching et Zheng Y. Liu, and *D. yushanensis* Ching et P. C. Chiu are much larger in size than *D. protobissetiana*, being 76–100-cm tall and having 40–50-cm-long lamina and 34–45-cm-long petioles (Ching & Zhang 1983, Ching & Liu 1984, Ching & Chiu 1987). *Dryopteris caudifolia* Ching et P. C. Chiu and *D. consimilis* Ching et P. C. Chiu have laminae with abruptly acute apexes (Ching & Chiu 1987). *Dryopteris lungjingensis* Ching et P. C. Chiu has ovate-deltoid laminae (Ching & Chiu 1987). *Dryopteris tieanzuensis* Ching et P.C. Chiu has papyraceous laminae (Ching & Chiu 1987). *Dryopteris glabrescens* Ching ex K. H. Shing et J. F. Cheng is similar to *D. sordidipes* in gross morphologies and has tripinnate laminae (Ching & Shin 1990). *Dryopteris pseudobissetiana* Ching ex K. H. Shing et J. F. Cheng has tripinnate laminae (Ching & Shin 1990). *Dryopteris quadrifida* Ching ex K. H. Shing et J. F. Cheng has 4-pinnate laminae (Ching & Shin 1990).

Dryopteris protobissetiana *as the key for understanding reticulate evolution in the* D. varia *complex*

Dryopteris protobissetiana is a key species for understanding morphological variation in the *D. varia* complex. Tagawa (1959) emphasized, in particular, the difficulty in distinguishing *D. varia*, *D. pacifica*, and *D. bissetiana* because of frequent morphologically intermediate forms between *D. varia* (sexual, apogamous) and *D. pacifica* (apogamous) and between *D. pacifica* and *D. bissetiana* (apogamous). Furthermore, Lee & Park (2013) reported frequent morphological variation between *D. bissetiana* and *D. saxifragivaria* (apogamous) and between *D. saxifragivaria* and *D. saxifraga* (sexual, apogamous). It has been considered that sexual species in the *D. varia* complex include only *D. varia* and *D. saxifraga* (Iwatsuki 1995, Ebihara *et al.* 2014), but no individuals with intermediate morphology between *D. varia* and *D. saxifraga* have been discovered. Individuals intermediate between *D. bissetiana* and other species are common, although the sexual form of *D. bissetiana* has never been reported. Recently, Hori *et al.* (2014) reported that the genome of *D. protobissetiana* was shared by apogamous species in the *D. varia* complex. If we suppose that *D. pacifica* originated as a hybrid between sexual *D. varia* and sexual *D. protobissetiana* and that *D. bissetiana* originated as a hybrid between sexual *D. saxifraga* and *D. protobissetiana*, we can much more readily explain the morphological variation in the *D. varia* complex.

The reason for the rarity of Dryopteris protobissetiana

Although we searched for additional specimens of *Dryopteris protobissetiana* among specimens of the *D. varia* complex from Yakushima and neighboring areas, we found them to be from only the southern part of Yakushima at an altitude of approximately 300 m, which indicated a narrow geographic range for *Dryopteris protobissetiana* in Japan. We propose two reasons.

First, the climate of Yakushima is warm and wet. Rain is important for sexual ferns to propagate, because sexual gametophytes need water for fertilization. Because southern Yakushima differs little from the northern part, we cannot readily explain why *D. protobissetiana* is limited to the southern part.

Strong volcanic activity near Yakushima may explain the distribution of the fern. Kikai-caldera, near Yakushima, produced a large pyroclastic eruption, the Koya pyroclastic flow, 7300 years ago (Geshi 2009). That pyroclastic flow and its deposits covered nearly the entire area of Yakushima and may have had an influence on the formation of unique floras on the island. In fact, only the southern part of Yakushima is believed to have escaped those volcanic events, and many plants are suspected of surviving there. Rare plants can be observed only in narrow areas in southern Yakushima. *Dryopteris protobissetiana*, like other rare plants on Yakushima such as *Arachniodes cavalerii* (Christ) Ohwi, *Polystichum eximium* (Mett. ex Kuhn) C.Chr., and *Pteris kawabatae* Kurata, might have also survived in the area (Iwatsuki et al. 1995).

Conservation of Dryopteris protobissetiana *on Yakushima*

Many species of ferns on Yakushima have been severely damaged by wild deer (*Cervus nippon yakushimae*). *Dryopteris protobissetiana* should be protected from damage by deer and from environmental change. We must collect more detailed information on the geographical distribution on Yakushima to take appropriate measures to conserve this rare fern.

Taxonomic Treatment

Dryopteris protobissetiana K. Hori et N. Murak., **sp. nov.** — Fig.3

Typus. Japan, Kagoshima, Yakushima, Mt. Myojo, approximately 300 m, on a rock cliff near a muddy road in a forest, *K. Hori 913* (MAK 410913), 7 Aug 2013 (holotype, MAK).

Diagnosis. ***Dryopteris protobissetiana*** K. Hori et N. Murak. is most similar to *D. bissetiana* (Baker) C. Chr. in having slightly bullate scales and a dark green lamina, but differs from *D. bissetiana* in having the margins of the upper pinnae flat and serrate at the apex and the margins of the laminae flat.

Plants terrestrial, evergreen; rhizome, petioles, stalk of the pinnae and rachises densely scaly; rhizome 2–3 cm long, 1–2 cm in diameter, erect or slightly ascending, leaves cespitose; petiole 10–27 cm long, 1–2 mm in diameter, yellowish green; scales lanceolate, apex filiform; scales on base of petioles flat, 4–8 mm long, black, translucent, margin barely toothed; scales on upper petioles and rachises bullate, 0.5–1 mm long, base brown, margin entire, apically black; lamina 2-pinnate, occasionally tripinnate at base, narrowly triangular, widest at base, gradually narrowing to apex, 13–33 cm long, 7–25 cm wide, dark green, soft coriaceous, surface shiny, margin flat; rachis yellowish green, 10–30 cm long; apex of pinnae obtuse to acute; lowest pinna 3–17 cm long, 1–6 cm wide; pinnae at middle of frond 4.5–13 cm long, 1–3 cm wide; upper pinnae 0.5–3 cm long, 0.3–0.7 cm wide, apical margin finely toothed; stalk of basal pinnae 0.2–1.0 cm long, upper pinnae sessile; lowest basiscopic pinnules on lowest pinnae 2–6 x 0.6–3 cm, longest but not markedly so; sori round, between margin and costa or relatively nearer to margin; indusia reniform or circular, margin entire or erose, translucent, approximately 1–1.5 mm in diameter; spores 64 per sporangium; chromosome number $2n = 82$, sexual diploid.

Habitat and distribution. *Dryopteris protobissetiana* is known from only four stations in southern Yakushima, Kumage, Kagoshima, Japan; on rock cliffs and stones in forest; 260–400 m.

Japanese name. Motoitachishida.

Key to the species of the *Dryopteris varia* complex in Japan

1a. Scales deflexed 2
1b. Scales ascending 3
2a. Lamina oblong .... *D. saxifraga*
2b. Lamina narrowly triangular .... *D. saxifragivaria*
3a. Lamina papyraceous; center of indusia often red .... *D. pacifica*
3b. Lamina herbaceous or leathery; center of indusia translucent 4
4a. Lamina herbaceous 5
4b. Lamina leathery 6
5a. Lamina narrowly triangular (length: width= 3: 2–2: 1); apex of pinnae curved, obtuse ... *D. kobayashii*
5b. Lamina widely triangular (length: width= 4: 3–3: 2); apex of pinnae straight, acute .... *D. sacrosancta*
6a. Lamina hard, pentagonal, lowest basiscopic pinnules on the lowest pinna markedly elongated ... *D. varia*
6b. Lamina soft, triangular, lowest basiscopic pinnules on the lowest pinna usually not markedly elongated, rarely markedly elongated 7
7a. Lamina dark green or greenish gray, widely triangular (length: width= 4: 3–3: 2), lowest basiscopic pinnules on the lowest pinna often not markedly elongated, sometimes markedly elongated; indusia often ciliate, sometimes entire .... *D. pacifica*
7b. Lamina dark green, narrowly triangular (length: width= 3: 2–2: 1), lowest basiscopic pinnules on the lowest pinna not markedly elongated; indusia entire 8
8a. Margin of lamina flat; margin of apex of upper pinnae serrate .... *D. protobissetiana*
8b. Margin of lamina incurved; margin of apex of upper pinnae entire .... *D. bissetiana*

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Appendix 1. Specimens examined.

Locality	Elevation	Habitat	Date	Collector & number	Herbarium & number	Chromosome number	Reproductive mode	DNA amount (pg.)	Ploidy
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Mt. Myojo	300m	on a rock cliff on roadside in forest	Aug. 7, 2013	*K. Hori 906*	MAK410906	$2n=82$	sexual	14.6	2x
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Mt. Myojo	300m	on a rock cliff on roadside in forest	Aug. 7, 2013	*K. Hori 907*	MAK410907		sexual	15.6	2x
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Mt. Myojo	**300m**	**on a rock cliff on roadside in forest**	**Aug. 7, 2013**	***K. Hori 913*, holotype**	**MAK410913**		**sexual**	**16.6**	**2x**
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Mt. Myojo	300m	on a rock cliff on roadside in forest	Aug. 7, 2013	*K. Hori 914*	MAK410914		sexual	15.1	2x
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Mt. Myojo	300m	on a rock cliff on roadside in forest	Aug. 7, 2013	*K. Hori 915*	MAK410915		sexual		
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Mt. Mocchomu	390m	on a side of stone near a road in forest	Aug. 8, 2013	*K. Hori 916*	MAK410916		sexual	15.2	2x
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Mt. Mocchomu	390m	on a side of stone near a road in forest	Aug. 8, 2013	*K. Hori 917*	MAK410917		sexual	16.1	2x
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Mt. Myojo	265m		Oct. 10, 2006	*S. Tagane TF293*	TNS763335		sexual		
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Koshima			Oct. 4, 2006	*S. Tagane TF269*	TNS763312		sexual		
Japan, Kyushu, Kagoshima, Kumage, Yakushima, Hanaage river			Aug. 17, 1982	*T. Nakaike s. n.*	TNS580903		sexual		